Marked-Up Version of Specification

This application [claims priority to] is a division of co-pending U.S. Application No. 09/268,787, filed 3/16/1999, which is a continuation-in-part of U.S. Application [Serial] No. 08/994,719, filed 12/19/1997 (now U.S. Patent No. 5,908,626), which is a continuation-in-part of U.S. Application [Serial] No. 08/719,331, filed 9/25/1996 (now U.S. Patent No. 5,723,125) which is a continuation-in-part of U.S. Application [Serial] No. 08/579,211, filed 12/28/1995 (now abandoned), and to which priority is claimed under 35 U.S.C. §120.

To overcome these disadvantages, one can modify the molecule to increase its circulation half-life or change the drug's formulation to extend its release time. The dosage and administration frequency can then be reduced while increasing the efficacy. Efforts have been made to create a recombinant IFNα-gelatin conjugate with an extended retention time (Tabata, Y. et al., *Cancer Res.* 51:5532-8, 1991). A lipid-based encapsulated IFNα formulation has also been tested in animals and achieved an extended release of the protein in the peritoneum (Bonetti, A. and Kim, S. *Cancer Chemother Pharmacol.* 33:258-261, 1993).

Immunoglobulins of IgG and IgM class are among the most abundant proteins in the human blood. They circulate with half-lives ranging from several days to 21 days. IgG has been found to increase the half-lives of several ligand binding proteins (receptors) when used to form recombinant hybrids, including the soluble CD4 molecule, LHR, and the IFN-γ receptor (Mordenti J. *et al., Nature*, 337:525-31, 1989; Capon, D.J. and Lasky, L.A., U.S. Patent number 5,116,964; Kurschner, C. *et al., J. Immunol.* 149:4096-4100, 1992). The invention relates to using IFNα-Fc hybrids, which may or may not include peptide linkers between the IFNα and the Fc portion, for treatment of tumors.

Summary of the invention

The present invention relates to IFN-Fc hybrids and their use in treating tumors. The IFN hybrids can be IFNα-Fc or IFNβ-Fc hybrids. The IFNα-Fc or IFNβ-Fc in the hybrid includes variants, including the IFNβ variant in BETASERON™. The hybrids preferably (but not necessarily) include peptide linkers between the IFN and the Fc portion. These linkers are preferably composed of a T cell inert sequence, or any non-immunogenic sequence. The preferred Fc fragment is a human immunoglobulin Fc fragment, preferably the γ4 chain. The γ4 chain is

In the $2.5X10^6$ and $0.5X10^6$ groups, the take rate reached 60% by the end of the nine and half weeks. The tumors did not kill the mice and there was no sign of metastases.

Thus, it is concluded that a subcutaneous inoculation of $1.25X10^7$ Daudi Burkitt lymphoma cells will yield about 80% tumor takes in about four weeks.

2. ***In vivo* antiproliferation Study**

1. Experiment with daily dosing

Thirty-two mice inoculated with $12.5X10^6$ Daudi Burkitt lymphoma cells were randomly assigned to one of four treatment groups as shown in Table 3. ROFERON® A (IFN-α-2a, Hoffmann La Roche, Nutley, NJ) and IFN-α(16)-2a-Fc (having the linker shown in SEQ ID NO:11) treatment began the day after tumor inoculation. All the animals were dosed daily subcutaneously over the scruff and the treatment continued for eight consecutive weeks. During the treatment period, animals were monitored every 3-4 days for tumor development, and tumor size was measured as above. After the treatment period, weekly observations were continued for additional six months for animals that were tumor free by the time when treatment stopped.

Blood was collected retro-orbitally 24 hours post the last dosing day, one, two and four weeks after termination of the treatment for IFN-α-2a-Fc and one, two and three weeks after termination of ROFERON® A treatment. Serum Interferon level was determined by ELISA.

Table 3. Dose, route and schedule

Group	Dose	Route of Administration	Schedule
Control	Diluent	s.c.	daily
ROFERON®	$1X10^6$ IU/100μl	s.c.	daily
A	$1X10^6$ IU/100μl	s.c.	daily
IFN-α-Fc	$1X10^5$ IU/100μl	s.c.	daily
IFN-α-Fc			

2. Effect of IFN-α on tumor take rate and tumor progression

Tumor development in different treatment groups is shown in Table 4. In control animals, the first tumor was detected 24 days after inoculation and within 6 days thereafter 7/8 (87.5%) of the animals had developed tumors. The average time of tumor detection was 25.1± 2.3 days (The mouse that developed a tumor at day 75 was not included.). In ROFERON® A treated animals, the first tumor became detectable 32 days after the inoculation. After another two weeks, 87.5% had developed tumors. The average tumor detection time was 39.6±4.7 days ($t > t_{0.05\ (12)}$, $P<0.05$). ROFERON® A delayed tumor development for about two weeks. IFN-α-2a-Fc treatment at both doses completely prevented the Daudi lymphoma from developing throughout the entire dosing period. At the lower dose, two mice developed detectable tumors at 2 and 19 days after cessation of the treatment. While all mice in $1x10^6$ IU/day group and the remaining six mice in $1X10^5$ IU/daily still remained tumor free six months post treatment. (Table 4). This experiment was repeated once with similar results, as shown in Table 4.

Table 4. Tumor Development in CB17/scid Mice (Exp.1.)

Mouse I.D.	Date of Inoculation	Date of Tumor Detection	Tumor Development Time (days)	Mean±S.D.
C* 116	5/27/98	6/20/98	24	
117	5/27/98	6/20/98	24	
125	5/27/98	6/20/98	24	
134	5/27/98	6/20/98	24	
114	5/27/98	6/20/98	24	
101	5/27/98	6/22/98	26	
119	5/27/98	6/26/98	30	25.1± 2.3
R* 133	5/27/98	6/28/98	32	
104	5/27/98	7/1/98	35	
103	5/27/98	7/6/98	40	
115	5/27/98	7/6/98	40	
110	5/27/98	7/7/98	41	
113	5/27/98	7/9/98	43	
128	5/27/98	7/12/98	48	39.6± 4.7

*C indicates a control

*R indicates that ROFERON® A was administered at $1X10^{6}$ IU/day

3. Effect of IFN-α on tumor growth rate

Once the tumor grew to about 1% of the mouse's body weight, tumor growth rate in control and ROFERON® A treated animals were very close. In control animals, average tumor volume increased 10 times in two weeks, while ROFERON® A treated mice showed a 9-fold increase.

Table 5. Tumor Take Rate in Different Treatments

Group	Treatment	Tumor Take Rate (%) (N=8)
Control	Diluent	100 (8/8)
ROFERON® A	$1X10^{6}$ IU /100ul	87.5 (7/8)
IFN α-2a-Fc	$1X10^{6}$ IU /100ul	0
IFN α-2a-Fc	$1X10^{5}$ IU /100ul	25.0 (2/8)

4. Quantitation of serum IFN-α level

Serum concentration of IFN-α and IFN-α-2a-Fc was determined by ELISA procedures. In ROFERON® A treated mice, IFN-α -2a was undetectable 24 hours after the last dose. In IFN-α-2a-Fc treated mice, serum IFN-α-2a-Fc concentration was 3.5 ug/ml for the $1x10^6$ IU/day group and 0.7 ug/ml for the $1x10^5$ IU/day group 22 days after termination of the treatment (Table 6). There was a decrease in serum concentration between 1 and 22 days after the end of the treatment. The data indicate that IFN-α-2a-Fc has a half-life of about one week in mice after being administered subcutaneously $1X10^6$ IU/day or $1X10^5$ IU/day for 8 weeks.

Table 6. Serum IFN-α-2a Level (μg/ml)

Treatment	Days Post Treatment Termination		
	1	8	22
IFN-α-2a-Fc $1X10^6$ IU	25.370±6.885	12.080±3.477	3.477±0.525
IFN-α-2a-Fc $1X10^5$ IU	2.766±1.138	1.549±0.536	0.691±0.141
ROFERON® A	Undetectable	Undetectable	Undetectable

5. Experiment with an increased-dosing-interval

In this experiment, ROFERON® A $1X10^6$ IU was given every 3 days and $1X10^6$ IU IFN-α-2a-Fc was dosed every three days and weekly. The results are shown in Table 7. ROFERON® A $1X10^6$ IU for 3 days failed to show any protection against tumor formation as compared to the control animals in tumor volume and average time for tumor development, while $1X10^6$ IU IFN-α-Fc administered every three days and weekly effectively inhibited the tumor formation during the eight week treatment period. This inhibition extended to seven weeks after the treatment period.

Table 7.Tumor Development in animals with an increased dosing intervals

Treatment	Tumor Take Rate (%) (N=8)	Average Time for Tumor Development (days)
Control	100 (8/8)	21.1±1.1
ROFERON® A 10^6 IU/ 3 days	100 (8/8)	22.0±1.9
IFN-FC 10^6 IU/ 3 days	N/A	N/A
IFN-FC 10^6 IU/ weekly	N/A	N/A

7. Preliminary study with established Daudi Burkitt lymphomas

Two mice with well established 5-week-old Daudi Burkitt lymphomas were treated with IFN-α-Fc at10^6 IU/daily. After ten days, complete regression was observed in both of the animals (Table 8). Two other mice with established 6.5-week-old Daudi lymphomas were treated with 10^6 IU ROFERON® A every three days for eight weeks. In the latter mice, tumor volume decreased rapidly, declining from 2.7cm^3 and 4.6 cm^3 to 0.3 cm^3, a reduction of 89% to 94% in the first two weeks. Complete regression was not achieved.

Table 8. Tumor Regression in Control Mice

Mouse I.D.	Date	Tumor Volume (cm^3)
416	11/20/98	0.195 (7 mm X7.6 mm)
	11/24/98	0.161 (6.4 mm X7.6 mm)
	11/25/98	palpable
	11/26/98	palpable
	11/27/98	complete regression
453	11/20/98	0.858 (10 mm X 16 mm)
	11/24/98	0.393 (6.4 mm X 7.6 mm, 7.6 mm X7.6mm)
	11/25/98	palpable
	11/26/98	palpable
	11/27/98	palpable
	11/28/98	barely palpable
	11/29/98	complete regression

Summary and Conclusions

1. A murine human tumor xenograft model has been established by inoculating subcutaneously female CB17/scid mice (six and half weeks old) with $1.25X10^7$ Daudi Burkitt lymphoma cells in the lower right flank at a total volume of 100 μl.
2. ROFERON® A $1x10^6$ IU/day treatment delayed the Daudi B cell lymphoma development by two weeks ($t > t_{0.05\ (12)}$, $P<0.05$). IFN-α-2a-Fc $1X10^6$ IU/day completely inhibited the tumor formation throughout the entire dosing period and this inhibition has been extended to six months after termination of the treatment. Partial to full inhibition was also shown in the $1X10^5$ IU /day IFN-α-2a-Fc treated mice.
3. ROFERON® A $1x10^6$ IU/ 3 days treatment failed to show any protection against the tumor development whereas Daudi Burkitt lymphoma has been completely inhibited by either IFN-α-Fc at $1x10^6$ IU/3 days or the IFN-α-2a-Fc $1x10^6$ IU/ weekly, and inhibition continued for at least seven weeks after cessation of the treatment.
4. Preliminary data demonstrated that established, 5-week-old Daudi Burkitt lymphomas are completely regressed when treated with IFN-α-2a-Fc 10^6 IU/daily for ten days. A 90% reduction of tumor volume in 2 weeks is also achieved in Daudi Burkitt lymphomas which were treated with 10^6 IU ROFERON® A/every 3 days for seven weeks before the IFN-α-2a-Fc treatment started.
5. IFN-α-2a-Fc has a half-life of about one week, when administered subcutaneously $1X10^6$ IU/day or $1X10^5$ IU/day for eight weeks.

It should be understood that the terms and expressions used herein are exemplary only and not limiting, and that the scope of the invention is defined only in the claims which follow, and includes all equivalents of the subject matter of those claims.

To overcome these disadvantages, one can modify the molecule to increase its circulation half-life or change the drug's formulation to extend its release time. The dosage and administration frequency can then be reduced while increasing the efficacy. Efforts have been made to create a recombinant IFNα-gelatin conjugate with an extended retention time (Tabata, Y. et al., *Cancer Res.* 51:5532-8, 1991). A lipid-based encapsulated IFNα formulation has also been tested in animals and achieved an extended release of the protein in the peritoneum (Bonetti, A. and Kim, S. *Cancer Chemother Pharmacol.* 33:258-261, 1993).

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Thus, it is concluded that a subcutaneous inoculation of $1.25X10^7$ Daudi Burkitt lymphoma cells will yield about 80% tumor takes in about four weeks.

2. ***In vivo* antiproliferation Study**

1. Experiment with daily dosing

Thirty-two mice inoculated with $12.5X10^6$ Daudi Burkitt lymphoma cells were randomly assigned to one of four treatment groups as shown in Table 3. [Roferon] ROFERON® A (IFN-α-2a, Hoffmann La Roche, Nutley, NJ) and IFN-α(16)-2a-Fc (having the linker shown in SEQ ID NO:11) treatment began the day after tumor inoculation. All the animals were dosed daily subcutaneously over the scruff and the treatment continued for eight consecutive weeks. During the treatment period, animals were monitored every 3-4 days for tumor development, and tumor size was measured as above. After the treatment period, weekly observations were continued for additional six months for animals that were tumor free by the time when treatment stopped.

Blood was collected retro-orbitally 24 hours post the last dosing day, one, two and four weeks after termination of the treatment for IFN-α-2a-Fc and one, two and three weeks after termination of [Roferon] ROFERON® A treatment. Serum Interferon level was determined by ELISA.

Table 3. Dose, route and schedule

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3. [Roferon] ROFERON® A $1x10^6$ IU/ 3 days treatment failed to show any protection against the tumor development whereas Daudi Burkitt lymphoma has been completely inhibited by either IFN-α-Fc at $1x10^6$ IU/3 days or the IFN-α-2a-Fc $1x10^6$ IU/ weekly, and inhibition continued for at least seven weeks after cessation of the treatment.
4. Preliminary data demonstrated that established, 5-week-old Daudi Burkitt lymphomas are completely regressed when treated with IFN-α-2a-Fc 10^6 IU/daily for ten days. A 90% reduction of tumor volume in 2 weeks is also achieved in Daudi Burkitt lymphomas which were treated with 10^6 IU [Roferon] ROFERON® A/every 3 days for seven weeks before the IFN-α-2a-Fc treatment started.
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